Report on:

GAS CHROMATOGRAPHIC TECHNIQUES FOR THE IDENTIFICATION AND STUDY OF NUCLEOSIDES

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GAS CHROMATOGRAPHIC STUDIES ON PURINE AND PYRIMIDINE BASES

The development of gas chromatography and its successful application to many important analytical problems in the field of biochemistry, such as steroids, fatty acids, and amino acids, suggests that a similar method for the analysis of purine and pyrimidine bases or the nucleosides might be developed. Of particular interest here would be the determination of purine and pyrimidine base ratios in the hydrolysates of nucleic acids (DNA and RNA).

Methods of analyzing these components by column, paper, and ion exchange chromatography have already been developed (2,3,7), but the sensitivity and speed of gas chromatography offers definite advantages. However, before gas chromatographic analysis of the purine and pyrimidine bases or the nucleosides can be accomplished, these compounds must first be converted to a suitable volatile derivative. It has been found, "... that free purines and pyrimidines can be methylated on their ring nitrogens to yield satisfactory volatile derivatives for gas chromatography." The major problem encountered by MacGee (5) was that of multiple derivatives for adenine, guanine, and cytosine. Hancock and Coleman (4)

reported the analysis of the ribo- and deoxyribo-nucleosides using the trimethylsilyl derivatives. This method will require much refinement of techniques, as the peaks obtained for the derivatives were unsymmetrical, and in some instances extraneous peaks were obtained. Miles and Fales (6) chromatographed derivatives of the nucleosides, "... in which the OH or NH_2 groups were blocked by combinations of acetylation, methylation, or isopropylidene formation." Similar chromatographic problems such as unsymmetrical and tailing peaks were encountered as cited in previous references.

The purpose of this investigation is to develop a suitable method for quantitative analysis of the base composition of nucleic acids. The solution to this problem may lie in a volatile derivative of the purine and pyrimidine bases, or in derivatives of the nucleosides.

The present report summarizes the preliminary investigation of the trifluoroacetyl derivatives of the purine and pyrimidine bases and the more successful studies of the methylation of the ring nitrogens of the purine and pyrimidine bases, using tetramethylammonium hydroxide (TMAH) and derivative formation in a flash heater at 350°C.

EXPERIMENTAL

Apparatus. An F and M Model 402 Biomedical gas chromatograph equipped with a dual hydrogen flame detector, was used in this study.

Column packings used were 1% w/w Diethyleneglycol succinate (DEGS), coated on acid-washed Chromosorb W

(60/80 mesh), and 1% w/w Carbowax 20M-TPA coated on acid-washed Chromosorb W (60/80 mesh). Glass columns were U-shaped, 1 meter long, with an internal diameter of 4 mm. A heated coil made with a glass tube (4 mm i.d.) spiraled (3" x 3/8" i.d.) around a cartridge heater (250 watts) was used in place of the flash heater.

Area determinations of the chromatographic peaks were made with a Type 16, Ott-Planimeter.

Reagents. The methyl stearate (used as the internal standard), 1,3-dimethyl uracil, and the purines and pyrimidines (adenine, guanine, uracil, thymine, and cytosine) were purchased from Mann Research Laboratories, New York. Trifluoroacetic anhydride was obtained from Eastman Organic Chemicals, Rochester, New York. Tetramethylammonium hydroxide (TMAH) was purchased from K and K Laboratories, Plainview, New York.

Trifluoroacetylation Procedure. Trifluoroacetic anhydride (1 ml.) was added to a solution of diethyl ether (4 ml.) containing 10 mg. of uracil. The resultant solution was sealed with a teflon lined cap in a heavy-wall centrifuge tube and heated at 150°C. for 5 minutes. The solution, upon cooling, was chromatographed on the 1% DEGS column, programming from 150° to 250°C. at 7.8°C./min.

Methylation Procedure.

Procedure A. The purine or pyrimidine base (ca. 10 mg.) was dissolved in a solution of 5 ml. of methanol

(A.C.S. grade) containing 83.3 mg. of TMAH. The resulting solution was injected into the spiral glass heater at 350°C., and chromatographed on a 1% Carbowax 20M-TPA column, programming the column temperature from 135° to 210° at 5°C./min. Immediately following injection of the sample, a 5 min. isothermal period was employed.

Procedure B. Trifluoroacetic anhydride (1 ml; 100 molar excess) was added to 10 mg. of purine or pyrimidine base and heated in a sealed tube at 150° for 5 min. The solvent was removed with a rotary evaporator. The product was then dissolved in 5 ml. of methanol (A.C.S. grade) containing 83.3 mg. of TMAH, and this solution was chromatographed under the conditions given in Procedure A.

Determination of Optimum Temperature for the Spiral Glass Heater. Thymine was methylated as outlined in Procedure A, and 2.5 mg. of methyl stearate added as an internal standard. The temperature of the glass heater was set at 300°, 350°, 375°, and 400°C.; injections of the thymine solution were made, and chromatographed under the conditions given in Procedure A. The ratio of the thymine peak relative to the methyl stearate peak was determined.

Determination of the Yield of Methylated Uracil. Uracil (10 mg.) was dissolved in a solution of 5 ml. of methanol (A.C.S. grade) containing 83.3 mg. of TMAH and 2.5 mg. of methyl stearate. Also, the pure derivative, 1,3-dimethyl uracil (10 mg.) was dissolved in a solution of 5 ml. of

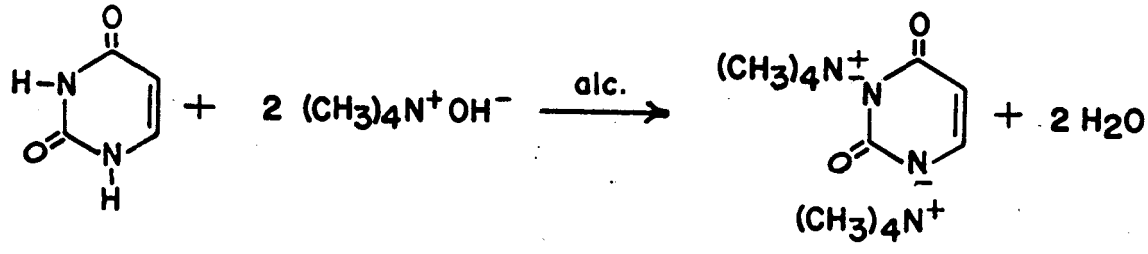
methanol (A.C.S. grade) containing 2.5 mg. of methyl stearate. The two solutions were chromatographed under the conditions given in Procedure A. Four determinations of each sample were made, and the yield calculated by the following formula:

$$\% \text{ yield} = \frac{\text{R.A. uracil/moles of uracil}}{\text{R.A. 1,3-dimethyl uracil/moles of 1,3-dimethyl uracil}} \times 100$$

Where R.A. (relative area) =

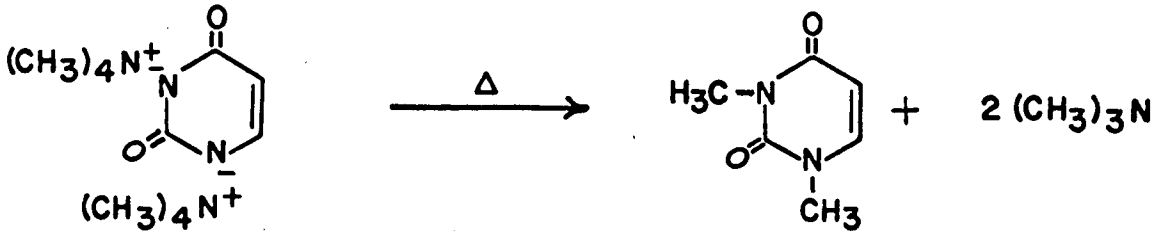
$$\frac{\text{peak area of uracil or uracil derivative}}{\text{peak area of internal standard}}$$

The purines and pyrimidines react with TMAH to form TMA salts: (5)



URACIL TMAH TMA SALT OF URACIL

The salts partially decompose upon heating at temperatures greater than 250°C. to give methylated derivatives:



TMA SALT OF URACIL

1,3-DIMETHYL URACIL

TRIMETHYL AMINE

The mechanism of this reaction has not been investigated, however, this type of reaction is generally considered to be ionic. Encouraging results were obtained on methylation of the pyrimidine bases with TMAH, as both thymine and uracil give single peaks. These peaks were both separable and symmetrical (Figure I). Methylation of adenine, guanine, and cytosine gave a multiplicity of peaks for each compound. These peaks are apparently due to different reactions involving the exocyclic amino groups (Figure II).

RESULTS AND DISCUSSION

Preliminary investigations on trifluoroacetylation of uracil yielded no positive results. The chromatogram showed one broad, tailing peak with a retention temperature of approximately 230°C.

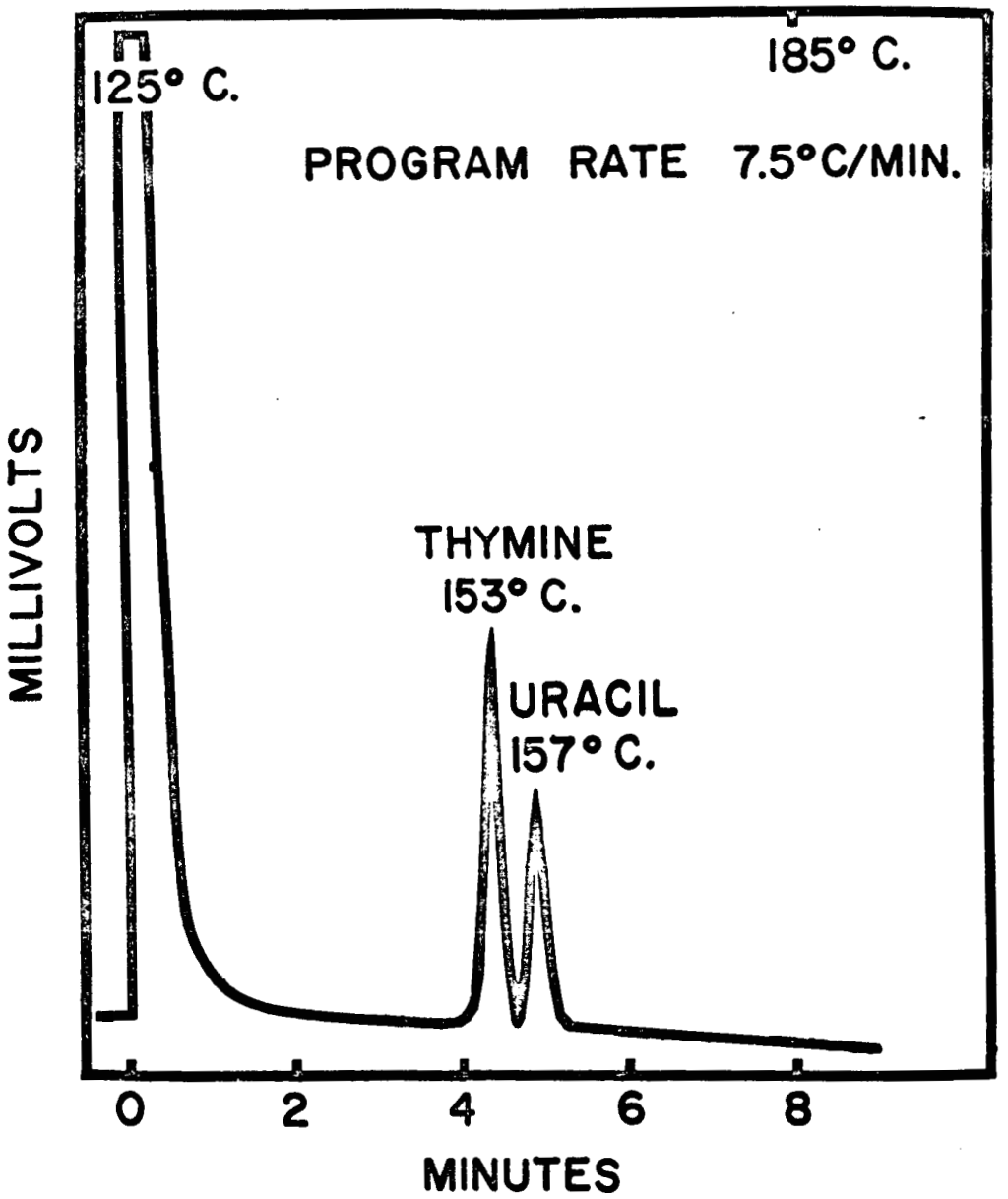
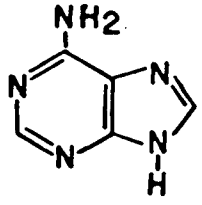


Figure I

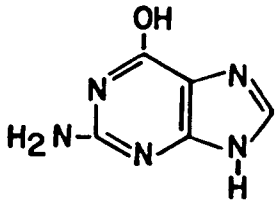
SEPARATION of THYMINE and URACIL

Sample size : 6 µl., 7.5 µg. of each pyrimidine



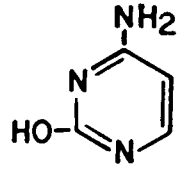
ADENINE

$C_5H_5N_5$



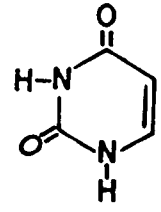
GUANINE

$C_5H_5N_5O$



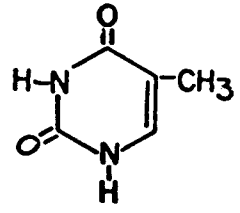
CYTOSINE

$C_4H_5N_3O$



URACIL

$C_4H_4N_2O_2$



THYMINE

$C_5H_6N_2O_2$

Figure II

STRUCTURES AND FORMULAS OF PURINES AND PYRIMIDINES

To overcome the side reactions involving the exocyclic amino group, Procedure B was used to trifluoroacetylate this amino group prior to methylation with TMAH. The results at this point indicate that the ratio of the main peak for adenine to the extraneous peaks increases when reacted in this way. Further investigations are underway to define these effects.

The optimum temperature of the spiral glass heater for the decomposition of the TMA salts of thymine was 350°C. (Figure III).

The per cent conversion of the TMA salt of uracil to 1,3-dimethyl uracil in the glass heater was 80% with a relative standard deviation of 2.7%.

SUMMARY

The conversion of purine and pyrimidine bases to alkylated (methyl) derivatives appears to be, at present, the more suitable method of derivatization, as indicated by the reproducible (±2.7%) conversion of uracil(80%) to the methyl derivative. However, the multiple peaks obtained for adenine, guanine, and cytosine, point out the need for more evaluation of the reaction parameters.

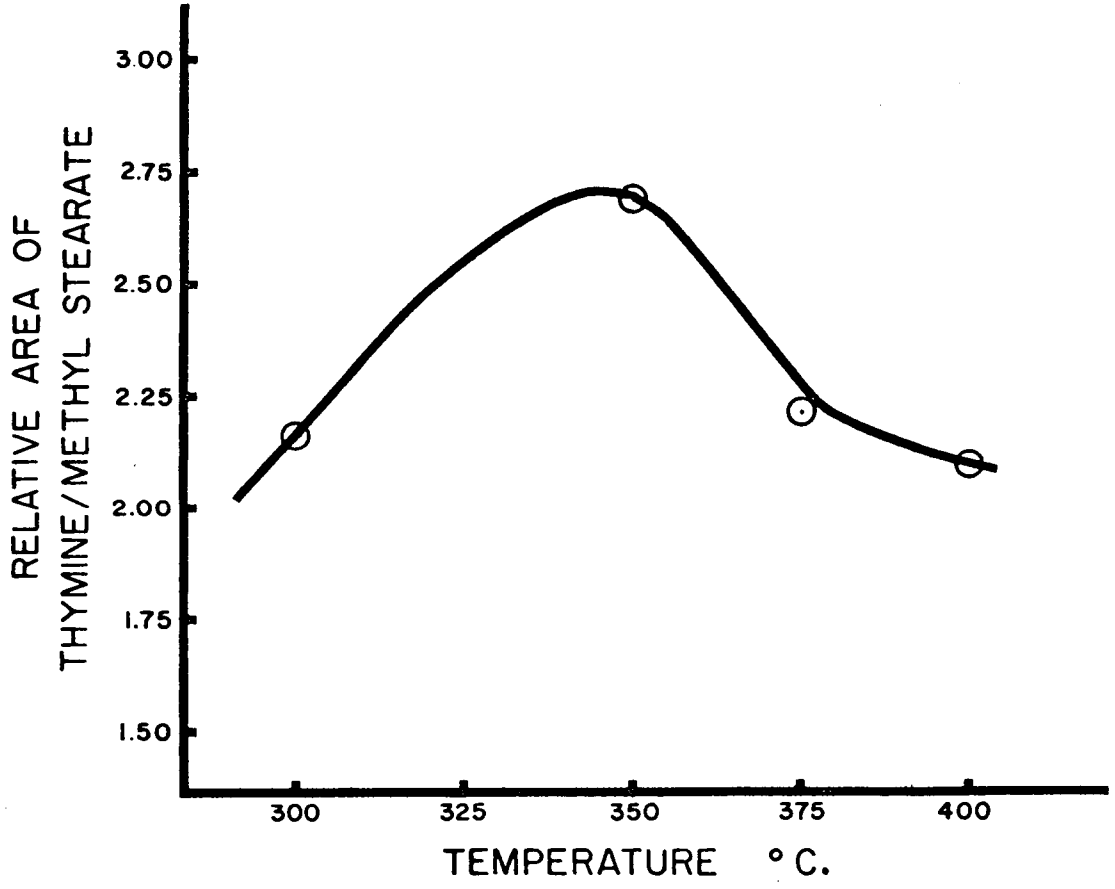


Figure III

TEMPERATURE EFFECT OF THE GLASS HEATER

PROPOSED RESEARCH

Experiments which are planned to delineate some of the possible choices for derivative formation are as follows:

Purine and Pyrimidine Bases.

1. Sealed tube dissociation studies on the TMA salts of the purine and pyrimidine bases prior to their chromatography.

The dissociation of these salts occurs in the spiral glass heater. Since the carrier gas is continually purging the glass heater, no control of the time required for this dissociation can be achieved. Therefore, the use of a sealed chamber that can be heated to specified temperatures (250°C. to 400°C.), which can be maintained for a definite interval of time will allow a more complete evaluation of these parameters.

2. Alkylation of the purine and pyrimidine bases with tetraethyl- or tetrapropylammonium hydroxide.

Since alkylation of these bases with tetramethylammonium hydroxide yielded one or two major and several minor peaks for adenine, guanine and cytosine, the reaction with tetraethyl- or tetrapropylammonium hydroxide may clarify the type of reaction involved, and help define the reaction conditions for optimum derivative formation and determine whether the reaction mechanism is free radical or ionic.

3. Alkylation of the purine and pyrimidine bases with tetramethyl- or tetraethyl lead and/or acylation of the exocyclic amino groups with lead tetraacetate.

In the event that the preceding alkylation studies should point out the need for a different type of alkylating agent or a combination of alkylation and acylation steps, the reaction of the purines and pyrimidines with tetramethyl- or tetraethyl lead combined with lead tetraacetate will be evaluated.

4. Free radical silation of the purine and pyrimidine bases with bis(trimethylsilyl) mercury.

Bis(trimethylsilyl) mercury decomposes under heat and light to give trimethylsilyl radicals:

$$(Me_3Si)_2Hg \longrightarrow 2\ Me_3Si\cdot + Hg$$

These free radicals, upon formation, may react reproducibly with the ring nitrogens in addition to exocyclic amino groups and hydroxyl groups, to yield trimethylsilated derivatives of the purine and pyrimidine bases as well as the nucleosides. The mercury compound ($(Me_3Si)_2Hg$) is synthesized by treating mercury amalgams with trimethysilyl chloride (1).

Nucleosides and Nucleotides.

Trimethylsilation of the nucleosides and nucleotides with hexamethyldisilizane and trimethylchlorosilane.

The reaction of hexamethyldisilizane and trimethylchlorosilane with carbohydrates and phosphorylated carbohydrates yields volatile derivatives suitable for gas chromatography.

Evaluation of the reaction of the nucleosides with bis(trimethylsilyl) mercury might lead to an improved method of derivative formation.

To this date the trimethylsilyl derivatives formed from the nucleosides have been difficult to chromatograph and completeness of derivative formation has not been demonstrated (4).

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